

Research Report

**Monosynaptic excitation of preganglionic vasomotor neurons
by subretrofacial neurons of the rostral ventrolateral medulla**

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Abstract

Extracellular single unit recordings were made from barosensitive neurons in the subretrofacial nucleus (SRF) of the rostral ventrolateral medulla in chloralose-anaesthetised cats. At the same time, single preganglionic neuron activity was recorded from filaments of the cervical sympathetic trunk (CST); barosensitive units were selected for study. Evidence for monosynaptic connections between the two neuron groups was sought by cross-correlation analysis of their ongoing activity. Cross-correlograms of 16/16 SRF/CST neuron pairs showed a broad peak (100–200ms wide), reflecting the synchronising action of arterial baroreceptors on both neurons' activity. Two of the 16 cross-correlograms additionally showed a robust, statistically significant, narrow peak of a single 2 ms bin width, providing the first physiological demonstration that ventrolateral medullary neurons monosynaptically excite preganglionic sympathetic neurons. Deductions are made about the strength, convergence and divergence of the connection.

Key words: Medulla oblongata; Sympathetic; Vasomotor; Preganglionic neuron; Monosynaptic; Blood pressure

1. Introduction

Neurons of the rostral ventrolateral medulla (RVLM) are a major source of tonic and reflex drive to sympathetic vasomotor nerves [7,10,24,32]. In cats, these neurons form a compact cell column – the subretrofacial (SRF) nucleus [10,31]. Many, though by no means all of these cells, synthesize catecholamines, and constitute part of the C1 adrenaline neuron group [10,19,26,31,32]. There is much anatomical [2,11,27,32] and physiological [3,5,23,29,35] evidence that SRF neurons, or RVLM neurons with the appropriate properties, send axons to the sympathetic preganglionic nuclei of the spinal cord. There, they are believed to contact sympathetic preganglionic neurons; but only limited *anatomical* evidence [27,34], and no direct *physiological* evidence, supports the view that they do so *monosynaptically*. Nor is it clear how widespread or selective such monosynaptic connections might be.

The present study has sought electrophysiological evidence that SRF neurons and sympathetic pregan-

glionic neurons are monosynaptically connected, using the method of cross-correlation [17].

2. Materials and methods

2.1. General

Experiments were performed on 7 cats of either sex (3.1–3.5 kg), anaesthetised with ketamine hydrochloride (15 mg/kg i.m.; Parke-Davies) and α -chloralose (45 mg/kg i.p.). Supplementary doses of 5–10 mg/kg α -chloralose were given i.v. when necessary. After tracheostomy and cannulation of the left jugular vein and right femoral artery (for drug administration and blood pressure monitoring, respectively), animals were paralysed with pancuronium bromide (Organon, 0.8 mg/h, i.v.) and artificially ventilated to keep end-tidal CO₂ at 4–4.5 vol.%. In some cases oxygen was added to the inspired air. Intra-oesophageal temperature was measured and maintained close to 38 deg. by a servo-controlled heating blanket. The ECG was recorded conventionally from needle electrodes. The urinary bladder was cannulated transurethrally or suprapubically in order to measure urine production and control fluid balance. Full details of general procedures and criteria for adequate anaesthesia are given elsewhere [6]. The experiments were approved by the local animal care committee of the state administration and were conducted in accordance with German Federal Law.

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2.2. Surgery

The animal was fixed supine in a stereotaxic frame and then the cervical sympathetic trunk (CST) and medulla oblongata were exposed by a ventral approach [24]. The left carotid sinus was prepared as a blind sac and cannulated via the external carotid artery, through which it could be inflated with saline by turning a tap connecting it to a pressure reservoir set between 180 and 250 mmHg. The common carotid artery was occluded with a pneumatic cuff during such tests, but was otherwise left open to the circulation. The left CST was prepared for dissection about 15 mm below the superior cervical ganglion and covered with mineral oil in a pool made from the skin flaps. A dam made from agar-soaked cotton wool separated the pool from the medulla.

2.3. Recording

After opening the perineurium of the CST, filaments containing either a single active unit, or a number small enough that the activity of single units could be discriminated with an amplitude window discriminator. Recordings were made differentially between a platinum wire electrode and an indifferent electrode in the nearby tissue.

The dura and arachnoid mater were removed from the ventrolateral medulla. A micropipette containing 0.1 M sodium glutamate was mounted in a micromanipulator and inserted approximately 0.7 mm beneath different points over the ventrolateral medullary surface on the left (ipsilateral) side. At these sites small quantities of glutamate (approximately 10–30 nl) were pressure-injected [21], and the responses of arterial pressure and CST unit activity noted. The region of medulla from which glutamate injections gave brisk increases in CST unit activity and blood pressure was selected for unit recording. Glass-insulated tungsten microelectrodes were inserted by a stepping motor into this region through small openings made in the pia, and neural activity recorded extracellularly by conventional means [22]. Brief carotid sinus inflations were used as a search stimulus to locate the subretrofacial nucleus, whose neurons are abruptly silenced by the procedure [22]. Discriminable single units which were silenced by sinus inflation were selected for further study. Recording conditions were usually stabilised by covering the medullary surface with agar.

After appropriate amplification and filtering, signals from both the CST and medullary unit recordings were fed into window discriminators and to a tape recorder. The discriminator output pulses, ECG, blood pressure and carotid sinus pressure were also recorded on tape for subsequent off-line analysis. ECG-triggered histograms were computed in 8 ms bins from the ongoing activity of CST units during the experiment, and this was used to identify their barosensitivity [6]. Throughout the recording period, action potential waveforms were passed through an analog delay line and displayed on a variable persistence storage oscilloscope, triggered by the discriminator pulse. The entire waveform of the triggering spike could then be seen and unit discrimination confirmed. All analysis was performed on discriminated single units.

2.4. Analysis

Using a personal computer-based analysis system (CARDS, S. Tiedemann), cross-correlograms and autocorrelograms were computed off-line from pulses discriminating SRF and CST neuron spikes. Cross-correlograms were constructed routinely by triggering on SRF unit spikes, and constructing a histogram of the CST unit's activity during a time sweep from 200 ms before to 600 or 800 ms after the trigger, in 2 ms time bins. The system did not allow retriggering during a sweep, so any additional trigger spikes that occurred during the sweep time would have been missed. To check

the effect of this, selected cross-correlograms were reconstructed in reverse by triggering on the usually slower-firing CST unit, and the sweep time was reduced to 300 ms, thus minimising the number of missed counts. Other than reversing the time axis, in no case did this materially affect the form of the correlation. Autocorrelograms were constructed by using the same spike as the trigger and the response. Since these should be symmetrical about the trigger point, no pre-trigger time was included.

Broad peaks were seen in all cross-correlograms (see Results). The latency of a broad peak was taken at the weighted mean (centre of gravity) of the portion of the peak which showed above the histogram's mean bin count. (Judgement was necessary in some cases to determine the appropriate edges of the peak). The same procedure was used to measure the latency of peaks in ECG-triggered histograms. Comparisons were made between the broad peak latency of each cross-correlogram and the time lag between the respective peaks of the corresponding SRF and CST units' ECG-triggered histograms (see text). The correlation coefficient of this relation for 16 neuron pairs was calculated by least-squares linear regression.

Narrow peaks of a single 2 ms binwidth were seen in some cross-correlograms (see Results). Three criteria were applied before accepting these as evidence for monosynaptic excitation. First, the peak should appear at a plausible latency: those occurring between 20 and 160 ms after the trigger spike were considered. Second, the probability of that bin count arising by chance out of the background was calculated from the Poisson distribution, as described by Abeles [1], taking the mean bin count of the flanking 100 ms as the reference background level. Given that 70 bins were being assessed, only *P* values less than 0.001 were considered significant. Third, cross-correlograms were constructed from three sequential time segments of the double recording, to check that the putative peak grew steadily, rather than being due to a sudden artefactual occurrence [30].

3. Results

3.1. CST units

Filaments containing either a single active unit, or a small number of active units from which single units could be discriminated, were dissected from the cervical sympathetic trunk (CST). Eighteen CST units were selected for further study, and these showed spontaneous activity of between 0.14 and 3.7 spikes/s (median 0.65). They were classified functionally on the basis of their cardiac or respiratory rhythmicity and their response to noxious stimuli, as detailed elsewhere [6]: none was found to fire in periods of activity and silence gated by the respiratory cycle; in ECG-triggered histograms of their ongoing activity, 16/18 units passed a stringent test for significant (i.e. strong) cardiac rhythmicity [6]; stimulation of cutaneous nociceptors in the head increased the activity of all but two of the units, one of which was unaffected and one inhibited. The latter unit (which also showed significant cardiac rhythmicity) was thereby classified as a Group II CST neuron (cutaneous vasoconstrictor-like) [6]. Fifteen units were classified as Group I CST neurons (muscle vasoconstrictor-like) and two units as Group IV CST neurons (function unclear) [6].

3.2. Medullary units

Single unit microelectrode recordings were then made from the appropriate region of the subretrofacial (SRF) nucleus, as defined by glutamate microinjections (see Materials and methods). Fourteen spontaneously active, barosensitive neurons were identified, using brief inflations of the carotid blind sac as the search stimulus: this silenced their activity in every case (Fig. 1A). The neurons showed ongoing activity of between 0.77 and 9.5 spikes/s (median 2.2) and were located between 0.5 and 1.1 mm from the ventral medullary surface. As expected, a degree of cardiac rhythmicity was always visible in ECG-triggered histograms constructed from their background activity (Fig. 1B). None showed periods of activity and silence corresponding to the respiratory cycle. SRF neurons were not tested for their response to noxious stimulation, because accompanying blood pressure changes compromised our ability to hold unit recordings.

3.3. Cross-correlations

Cross-correlograms with a 2 ms time resolution were constructed from the discharges of 25 medullary neuron/CST neuron pairs. Because of the low firing rates, particularly of the CST units, recording periods of around an hour were usually necessary to accumulate enough counts to show the features described below. Data from 9 neuron pairs were rejected because the total count amounted to less than 2 spikes/bin in the cross-correlogram. Of the 16 which were analysed further, 15 neuron pairs included Group I CST neurons and a single pair a Group II CST neuron.

3.4. Broad peaks

The general form of the surviving 16 cross-correlograms was a broad peak, corresponding to an increased probability of the CST unit firing after a medullary unit spike (Fig. 2). If long sweep times were recorded, the peak could be seen to recur about one cardiac cycle later, and in pre-trigger time about one cardiac cycle earlier than the original peak (Figs. 2A and 3B). It therefore seemed likely that the broad peaks were connected with the cardiac rhythmicity that both types of neuron display in their ongoing activity.

The timing of each SRF and each CST unit's cardiac-related activity was measured from the ECG-triggered histogram as the latency of the peak closest to the triggering R-wave. In 15/16 neuron pairs, the cardiac-related activity peak of the medullary unit occurred earlier in the cardiac cycle than that of the preganglionic neuron. For every neuron pair, the time lag between their respective cardiac-related peaks was compared with the latency of the corresponding broad

peak in the cross-correlogram (Figs. 3 and 4). In the single case where the CST unit led the SRF unit in the cardiac cycle, a peak in the cross-correlogram was seen at an equivalent negative value. As can be seen in Fig. 4, there was good overall agreement between the paired latency values, considering the limited accuracy of the underlying measurements ($r^2 = 0.90$).

3.5. Narrow peaks

Monosynaptic connections have been found elsewhere to appear in cross-correlograms as a sharp peak in the order of a millisecond wide [17], so we looked

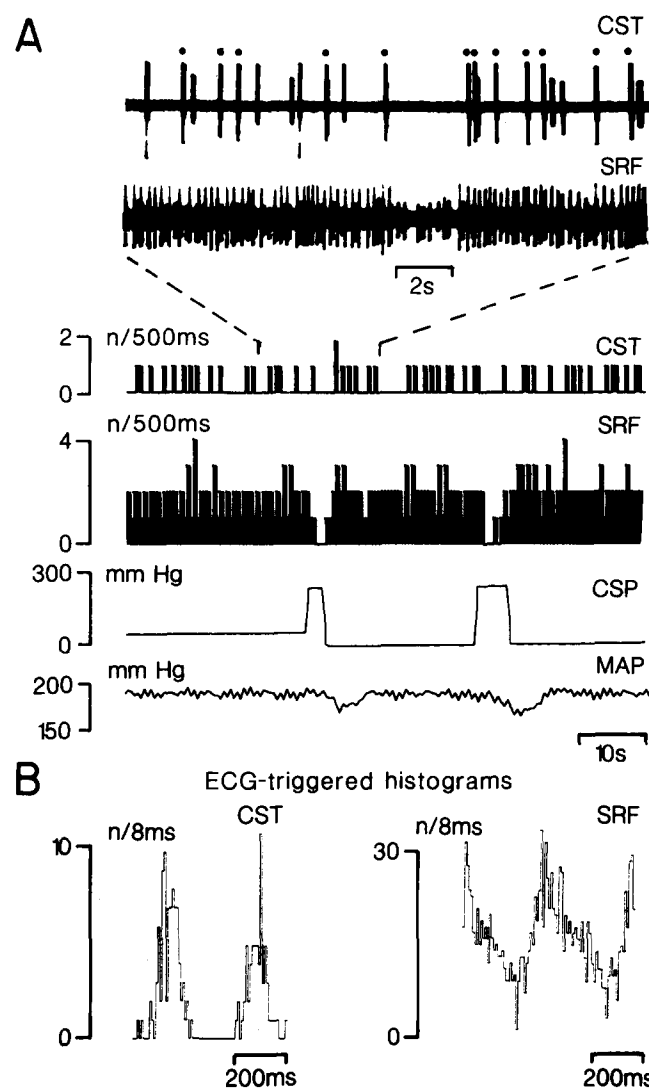


Fig. 1. A: chart record of discriminated single CST neuron and SRF neuron activities, carotid sinus pressure (CSP), and mean arterial pressure (MAP). The carotid sinus was twice inflated with saline to a pressure of 225 mmHg. The inset above shows CST and SRF unit recordings; the activity of a single CST unit (spikes marked by dots) was discriminated from that of other active units. B: ECG-triggered histograms of spontaneous SRF and CST unit activity (8 ms bins, 500 sweeps). The time axes cover approximately 2 cardiac cycles.

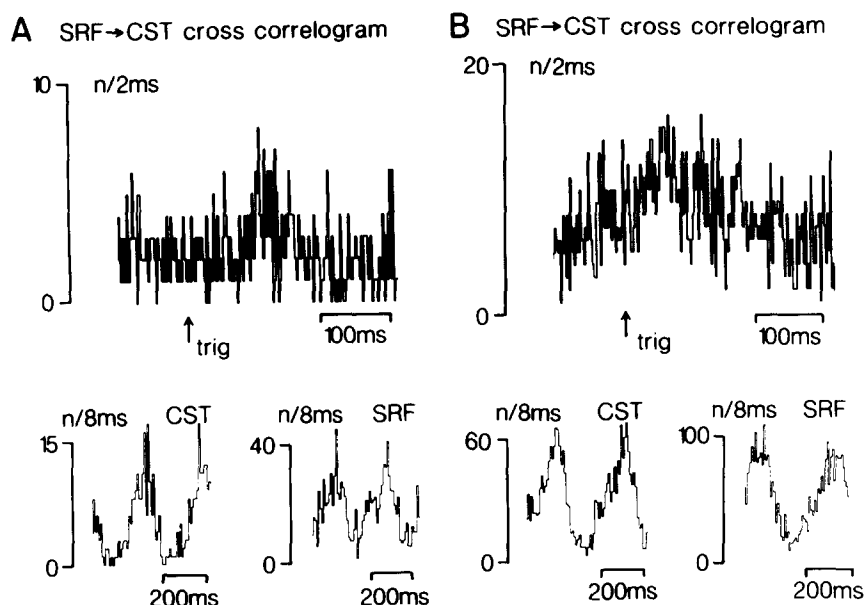


Fig. 2. Upper records, A and B: SRF-to-CST unit cross-correlograms (2 ms bins; 1600 and 1374 sweeps, respectively), each triggered by the SRF unit at the time indicated by the arrow. Note that both cross-correlograms show a broad peak, indicating increased CST unit activity following the SRF unit spike. Lower records: ECG-triggered histograms of the corresponding CST and SRF units' activity (8 ms bins, 1000 sweeps).

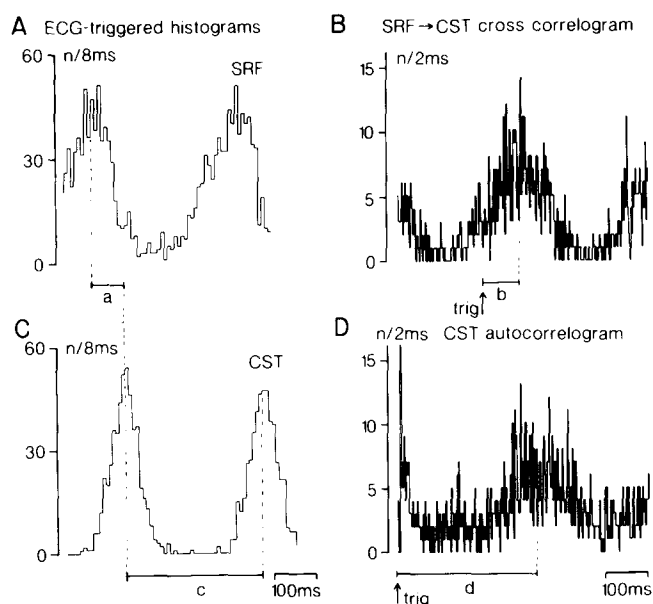


Fig. 3. A,C: ECG-triggered histograms of an SRF and a CST unit, respectively (8 ms bins, 1000 sweeps). 'a' represents the lag time between the mean latency of their respective peaks; 'c' represents the cardiac cycle time. B: cross-correlogram triggered by the SRF unit (at arrow), of the CST unit's activity (2 ms bins, 1173 sweeps). 'b' represents the mean latency of the broad peak. D: Autocorrelogram of the CST unit's activity, triggered at arrow (2 ms bins, 1000 sweeps). This unit fired frequent doublet spikes, giving rise to the narrow peak shortly after the trigger. The mean latency of the following broad peak is given by 'd'. Note the correspondence of 'a' with 'b', and of 'c' with 'd'.

within an appropriate latency range for peaks of similar width (i.e. probably confined to a single 2 ms bin). The latency range chosen was from 20 ms (estimated taking an upper overall conduction velocity limit of 10 m/s) to 160 ms, which would include most broad peak latencies. Two additional criteria were applied: firstly, the peak should exceed the surrounding bin counts by a significant amount and secondly, it should result from counts accumulated throughout the recording period (see methods). The second criterion controls against peaks from sudden, probably artefactual occurrences [30].

Two of the 16 cross-correlograms showed robust narrow peaks which satisfied those criteria (Fig. 5). They showed that the CST units fired with significantly increased probability at 32 ms ($P < 0.0001$) and 110 ms ($P < 0.0005$) after their respective SRF unit had fired (Figs. 5A and 5B, respectively). It may be noted that although these latency values fall within the range found for broad peaks as a whole, in both cases they occurred early in the broad peak of their own cross-correlogram (Fig. 4). Assuming conduction distances of 120 mm (bulbosplinal) plus 80 mm (preganglionic), the calculated overall conduction velocities for the two stages were 6.25 and 1.8 m/s.

Both narrow peaks exceeded their surrounding bin counts by a factor of approximately 3 (k value – an index of synaptic strength [13,17], and exceeded baseline values outside the broad peak by somewhat more.

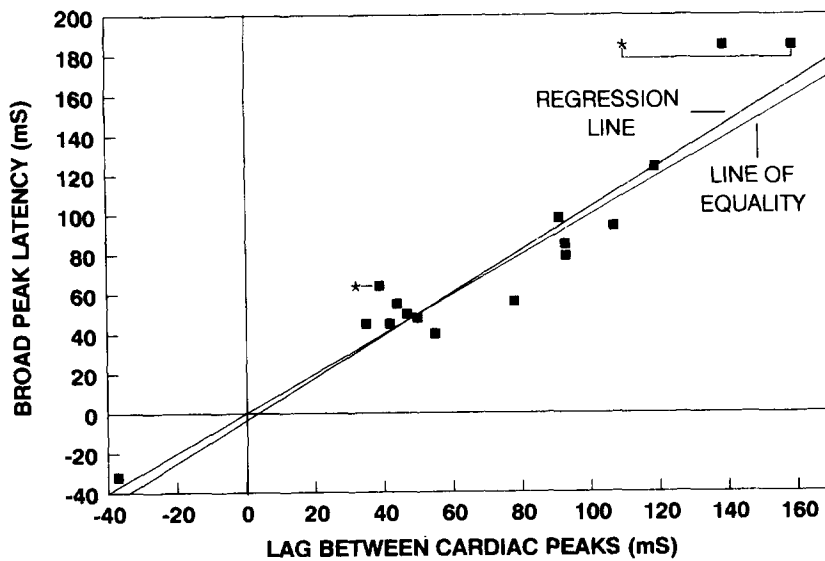


Fig. 4. Graph, for each of the 16 SRF/CST neuron pairs, of the mean broad peak latency in the cross-correlogram (c.f. 'b' in Fig. 3) vs. the time difference between the peaks of their respective ECG-triggered histograms (c.f. 'a' in Fig. 3). The regression line and line of equality are drawn. The 2 narrow peak latencies are also shown (asterisks) and linked to their respective broad peak values.

If we take baseline values of 7 and 2 for the lower traces in Figs. 5A and 5B, respectively, we can calculate that 22 and 11 extra spikes fell into the narrow peaks, and that 374 and 221 extra spikes fell into the

broad peaks, respectively. The probability of a CST spike being triggered monosynaptically by the SRF spike may be estimated from the number of sweeps as 0.63% and 0.46% in the two cases.

The CST units of both narrow-peaked cross-correlograms were functionally classified as Group I (muscle vasoconstrictor-like).

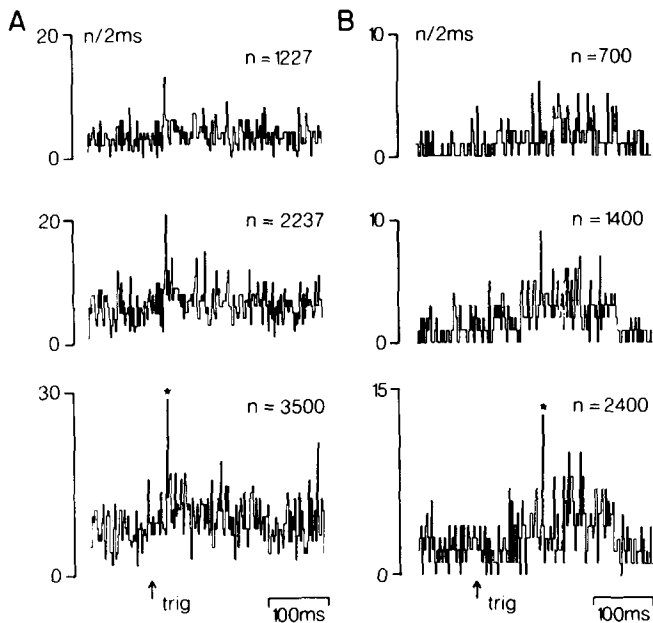


Fig. 5. The 2 cross-correlograms which showed significant narrow peaks (asterisked, lower traces), at 32 ms and 110 ms after the trigger spike, respectively. Segmentation of the data revealed (upper and middle traces) that the narrow peak grew steadily with increasing sweep numbers (*n* values given). In both cases the trigger was the SRF unit spike (time denoted by arrows) and the response was the CST unit spike.

4. Discussion

4.1. Narrow peaks

We interpret the presence in 2 cross-correlograms of robust, statistically significant, narrow peaks of appropriate latency, as due to monosynaptic excitation. Two alternative interpretations should first be considered, however: synchrony due to common inputs, and excitation via a disynaptic pathway.

The first of these proposals would require that the individual SRF and preganglionic neuron were synchronised, with millisecond precision, by a shared input. That millisecond precision would have to survive a bulbospinal link. However, the descending pathways from the medulla to preganglionic neurons show a range of conduction velocities, all of which are slow [8,14]: only small individual differences between axons would cause many milliseconds' scatter in bulbospinal conduction time. A cross-correlogram peak of 2 ms width could therefore not have resulted from inputs shared by the SRF and the CST neuron, except in the

unlikely events that the synchronising input arose either from a single neuron or from a population whose axonal conduction times were almost perfectly matched. On the other hand, common inputs could readily account for less well synchronised events such as broad cross-correlogram peaks.

For the second proposal (disynaptic excitation) to work, the excitatory drive would need to be transmitted strongly from the SRF neuron through 2 synapses, again without significant time dispersion. For similar reasons, to explain a narrow peak the hypothetical pathway would need to be either a single, powerful interneuron (which was itself powerfully driven by the SRF neuron) or a tight group of interneurons with virtually identical conduction times. Even minor temporal dispersion of EPSPs, which occurs in the tightest of disynaptic connections, broadens the peak, and more importantly, greatly reduces its height: much higher spike counts are thus needed for detection [17]. Disynaptic excitation would therefore also seem a most unlikely cause for the narrow cross-correlogram peaks found here, so we take them confidently as evidence for monosynaptic connections from SRF to preganglionic neurons.

The 2 narrow cross-correlogram peaks found in this study showed k values (peak to background ratio) of 3, indicating that the monosynaptic connections were relatively strong — considerably stronger, for example, than those to somatic motoneurons from bulbospinal respiratory neurons [13] or from muscle spindles [18], although weaker than those between retinal ganglion cells and lateral geniculate neurons [15]. As far as we are aware, no comparable data exist for inputs to preganglionic neurons, but the relative effect of an individual synapse is likely to be relatively greater on small cells such as these [17]. Stated another way, the probability with which a monosynaptic EPSP triggered a postsynaptic spike was about 0.5% in both cases. It should be noted, however, that additional, weaker connections could have remained undetected because of limited spike counts.

The present data provide physiological support for the anatomical finding that axon terminals labelled by anterograde transport of tracer from the RVLM make direct, asymmetric (presumed excitatory) synaptic contacts with preganglionic neurons in the rat spinal cord [27,37]. Those synapses primarily contact dendrites [27,37], and may contain glutamate [27] and/or the adrenaline synthesizing enzyme, phenylethanolamine N-methyl transferase (PNMT) [25]. Experiments showing transynaptic retrograde labelling of RVLM neurons by pseudorabies [33,34] or herpes simplex [19] virus injected into the adrenal gland or sympathetic ganglia also support the notion that SRF neurons make monosynaptic contact with preganglionic neurons.

4.2. Broad peaks

The presence of a broad peak in every cross-correlogram is a predictable consequence of the pulse rhythmicity that is common to both neuron types. This explanation is supported by the good match found between broad peak latency and the time lag between cardiac activity peaks for each neuron pair. The simplest interpretation of these findings is that each preganglionic cell is driven by a number of SRF neurons, which all fire at similar times in the cardiac cycle to the trigger cell. That number may be about 20, as judged from the respective broad peaks in the 2 cases where a narrow peak due to monosynaptic excitation by a single cell was available for comparison. (The extra spikes in the broad peak were about 20 times those in the narrow peak in each case.) The negative latency measured for one of the other broad peaks could also be explained in this model by the SRF trigger cell tending to fire later in the cycle than its fellows. Other pathways with pulse-related activity, such as the Raphe-spinal neurons with presumed sympathoinhibitory function [28], might also contribute to the cross-correlation.

The broad peaks detected in the present study are probably homologous to the slow waves which can be recorded by spike-triggered averaging of sympathetic whole nerve activity [3,4,29]. That relationship evidently persists between RVLM [3,29] or other [4,36] central neurons and the spontaneous bursts which remain in sympathetic nerve discharges after barodeneration. In the present study we did not disable the baroreceptors, however, so the activity pattern would have remained locked to the cardiac cycle.

4.3. Significance of findings

The present data provide the first physiological evidence for a monosynaptic excitatory connection from SRF to preganglionic neurons. Furthermore, the preganglionic neurons in question were identified by functional criteria as muscle vasoconstrictor-like (Group I CST neurons), so the connection probably belongs to a vasomotor pathway.

The monosynaptic connections found were individually quite strong, but were not universal, even between SRF and CST neurons preselected to be of the same functional type. The small proportion of monosynaptic connections found (2/16) is not surprising, given the limited degree of convergence at this synapse (see above), which would reduce the probability of finding a match. On the other hand, the divergence at this synapse must be considerable: a quarter of CST neurons [6], and by extrapolation perhaps 10,000 of the approximately 40,000 preganglionic neurons on each side of the body [16] may be muscle vasoconstrictor in function. These need to be supplied with an estimated

20 inputs each by the unknown fraction of SRF neurons (total c. 1000–1200 each side [31]), which drives muscle vasoconstrictor pathways [9,12,20] throughout the body [23]. In keeping with this point, it is known that some SRF neurons' axons may arborize extensively within the spinal cord [3].

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